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Functional analysis of genes in *Aedes aegypti* embryos

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INTRODUCTION

In recent years, RNA interference (RNAi) has proven to be an effective strategy for inhibiting gene function in many organisms. This protocol describes a method for knockdown of embryonic genes in *Ae. aegypti* embryos that involves microinjection of small interfering RNA (siRNA) designed to target a specific gene of interest. The following procedure outlines a strategy for siRNA design, microinjection, and measure of knockdown effectiveness.

MATERIALS

Equipment

Container with lid (tall enough to hold fly vial)

Cotton Balls

Coverslips

Dissecting Microscope

Double sided tape

Egg-collection chambers

Ethanol Bottle (70%)

Fly Vials

Forceps

Filter paper (Whatman reduced fiber)

Glass Capillary Tubes –Glass Thin Wall with Filament, World Precision Instruments, Inc, TW100F-4

Injection Block

Kimwipes

Light Source

Mesh Screen

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Conflicts of interest: none declared

Microbevelor (i.e. Sutter Instruments Co, K.T. Brown Type Micro-pipette Bevelor Model BV-10 with Extra Fine Diamond Abrasive Bevelor Plate Cat. # 104F)

Microinjection needles

Microinjector – Narishige IM 300 Microinjector

Microloader pipette tips (Eppendorf)

Micromanipulator (i.e. Narishige, Model MN-151)

Micropipetter (2 μl)

Mosquito mouth pipetter

Needle Puller (i.e. Sutter Instruments Co, Model p-87)

Needle storage container (i.e. Micropipette Storage Jar # E210, World Precision Instruments)

Paintbrush (fine, round)

Paper Towels

Razor Blades

Rubber Tubing

Slides

Stereoscope

Timer

Transfer Pipettes

Videocamera with monitor – (i.e. Sony Hyper HAD Model DXC-151A and Sony Trinitron Model PVM-1353MD)

Water Bottle

Reagents

Halocarbon Oil 27

Interfering RNA (6 μg/ μl)

Microinjection buffer <R>

Mosquitoes

METHOD

i. siRNA Design

When designing siRNAs for knockdown of *Ae. aegypti* genes, we follow the siRNA design guidelines posted by Ambion (http://www.ambion.com/techlib/tb/tb_506.html). In general, the sequence of a gene of interest can be pasted into the Ambion siRNA target finder, which will identify 21mers that begin with an AA dinucleotide. Per Ambion's suggestions, siRNAs should contain 30-50% GC content and lack stretches of four or more Ts or As. We recommend that multiple siRNAs be designed, as some may yield more effective knockdown than others. BLAST searches should be performed on siRNA sequences identified in order to ensure that they will only target the gene of interest. It is best to work with multiple siRNAs that yield high percentages of knockdown, as this helps to ensure that phenotypes identified are specific

to the gene being targeted and not the result of off-site targeting. Negative control siRNAs typically consist of scrambled nucleotide sequences of siRNAs used for knockdown experiments. BLAST searches should be used to confirm that these control siRNAs do not target any genes in the *Ae. aegypti* genome. We typically order siRNAs from Dharmacon (enzymatically synthesized, A4 purity), but several other reputable vendors exist. We also order biotin-labeled siRNAs, which allows for their tracking through streptavidin staining post-injection. It should be noted that although FITC-labeled RNAs were initially utilized in our studies, autofluorescence in the embryos made them difficult to track.

ii. Preparing Needles for Microinjections

1. Using gloves, pull capillary tubes to make microinjection needles. Although needle pulling settings are a matter of preference and machine type, the following settings for the Sutter Instruments P-87 model work well in our hands: Heat: 555, Vel: 75, Time: 150, Pul: 45, Pressure: 500. Pull 4-5 needles per condition. For example, if control siRNA and experimental siRNA will be used, pull 8-10 needles, which can be stored in a needle storage container until use. It is a matter of choice, but we recommend beveling needles at a 25 – 30° angle.
2. Using microloader pipette tips, add 1μL of siRNA per needle. It is useful to have extra needles on hand when you begin to microinject, as needles sometimes break or get clogged.

iii. Egg-collection

1. Obtain fly vials, large cotton balls, Whatman Filter Paper, a mouth pipette, forceps, a container with a lid, and a two-channeled timer. To make the mouth pipette, cut the tip off of a 10 mL automatic pipette tip and remove the filter end. Place a piece of mesh screen atop either end, and slide the rubber hose over it. Aspirate through the rubber hose without placing your lips onto it.
2. Place one large cotton ball in the bottom of the fly vial and dampen it with water. Wet circular filter paper (egg paper) such that the paper is damp, but not soaking wet. Using a 15 ml falcon tube, carefully press the egg paper into the bottom of the vial such that it is positioned over the cotton ball. It may be helpful to make multiple chambers.
3. Take care not to let mosquitoes escape during this step. Using the mouth pipette, collect ~6 female mosquitoes that were recently blood fed. Mouth pipette the mosquitoes into the chamber and quickly cover it with cotton so that the mosquitoes do not escape. Push the cotton plug down into the vial so as to prevent the females from flying, making them more likely to lay eggs. Place the chamber into a darkened container inside the insectary and set a timer for 30 min.
4. While waiting for the mosquitoes to lay their eggs, prepare coverslips to be used during microinjection. This is accomplished as follows: Wearing gloves, tear off a strip of double-sided tape and attach it to a finger on the palm side of your hand. Place coverslips along the edge of the tape allowing only 1 - 2mm of tape to stick onto the coverslip. Press gently but firmly. Peel the tape with coverslips off of your finger. Place the coverslips tape-side up on the bench. Using your razor blade, cut around the edges of the coverslips to remove excess tape. Place them tape-side up in a petri dish.
5. After 30 min have passed, use forceps to lift the cotton plug which facilitates viewing of the egg paper. If there are no eggs, then obtain new females and try again. If white eggs are visible, then release the adult females back into the cage. Keep the chamber bearing white eggs in the insectary for another 10 min, after which time they will have

begun to lightly pigment and are ready for microinjection. If you will be injecting multiple sets of eggs, then you may wish to set up another egg-collection.

iv. Microinjection

1. Obtain petri dishes, filter paper the size of the petri dish lid, a paint brush, a permanent marker, forceps, a water bottle, paper towels, a dissecting microscope, and a light source.
2. Line one petri dish with dampened filter paper. Remove egg paper from the egg-laying chamber and place it in the petri dish. Using a microscope and brush, align light grey to purplish eggs in a very straight line near the edge of the egg paper. The posterior (narrow) ends should all face in the same direction toward you. It is recommended that two or three sets of 10 eggs which have been aligned compactly (touching each other) be prepared. Work carefully but steadily. Eggs will need to be injected within approximately one hour of being laid. After this time, they will turn a dark purplish color, which serves as a signal that they are too old.
3. Once alignment is complete, dry the area immediately surrounding the aligned eggs by blotting gently with a paper towel. Place the double-sided taped coverslip tape-side down onto the eggs. Using gentle pressure, press down just enough to transfer the eggs to the double sided tape. Place the coverslip onto the microinjection stage.
4. Allow the eggs to desiccate until the egg appears dimpled (time varies, but roughly 20-60 sec). Watch the eggs very carefully during this step and don't let them overdry. Cover the eggs with Halocarbon Oil 27. Place the injection block on the stage of the injection microscope.
5. Inject at the posterior end of the embryo at roughly a 20° angle.
6. Following microinjection, remove the cover slip from the injection block and tap it onto a kimwipe to remove excess oil. Place the cover slip with injected eggs into a dampened paper towel-lined petri dish that will serve as a humidity chamber. Cover the eggs with the dampened paper towel.
7. For proper disposal of unused eggs, spray the eggs with 70% ethanol and discard them.

v. Analysis of Knockdown

Quantitative RT-PCR (qRT-PCR) is one of the most rapid methods for judging the effectiveness of siRNAs. When testing the effectiveness of new siRNAs, we typically prepare total RNA from mosquito embryos 24 - 72 hrs. post-injection. For qRT experiments, the *Ae. aegypti* housekeeping gene *rpS17* can be used as an internal standard for data normalization (Morlais *et al.*, 2003). *In situ* hybridization or immunohistochemical analysis of mRNA or protein levels can also be used to assess knockdown. These method offers a more spatial, though less quantitative, measure of knockdown.

TROUBLESHOOTING

1. *Problem: Poor egg laying (step iii);* Solutions: You may need to use multiple egg-collection chambers, especially if it has been five days post-blood feeding. If females are not laying, try different adult females. Be sure to blood feed your mosquitoes weekly if you are collecting eggs for microinjection. Prepare fresh cages of adults every two weeks.
2. *Problem: Needle breakage and clogging (step iv);* Solutions: Good needles are essential for proper injection. Sometimes the injection pressure can be adjusted to

compensate for a slight increase in tip opening due to breakage. However, if the tip becomes blunt or jagged, it is best to load a new one. Although we have not yet tried them, quartz needles may work well in conjunction with this protocol.

3. *Problem: Excess desiccation (step iv);* Solutions: To avoid over-drying eggs, the following solutions are recommended:

    a. Step iv.2: Keep the petri dish used to align eggs damp at all times. Align only as many eggs as your microinjection skill level permits.

    b. Step iv.4 of the microinjection procedure is critical. Do not over- or under-desiccate eggs. Over-desiccation leads to hard eggs and broken tips; under-desiccation leads to poor viability and needle flow back.

    c. Step iv.4: The technique we use employs halocarbon oil for the purpose of not allowing the eggs to over-desiccate. Some protocols use microinjection buffer, but depending on your skill level it will dry (and consequently so will the eggs).

    d. Step iv.6: Keep the filter paper lining petri dishes damp at all times.

    e. Step iv: Some labs perform their injections in a room equipped with a humidifier, which helps to prevent the eggs from drying too quickly.

4. Problem: Microinjection difficulties due to the use of improperly aged embryos (step iv); Solutions: Young, unpigmented eggs will be very delicate and injecting these eggs will result in decreased viability. On the other hand, if you inject after the eggs are highly pigmented, you will likely see an increase in needle breakage and may miss the syncitial blastoderm stage.

5. Problem: Microinjection frustration for the beginner (step iv); Solutions: It can be helpful to practice injecting with a colored dye. We also recommend working in pairs so that one person can align eggs while the other injects.

DISCUSSION

In recent years, RNA interference (RNAi) has proven to be an effective strategy for inhibiting gene function in many organisms. Here, RNAi, which has been used successfully in mosquito larvae and adults (Blitzer *et al.*, 2005; Luna *et al.*, 2007), is combined with microinjection techniques used for germline transformation of *Ae. aegypti* (Lobo *et al.*, 2006; Jasinskiene *et al.*, 2007). We have used the methodology described here to knock down the *Ae. aegypti frazzled (fra*, Clemons et al., in preparation) and *semaphorin-1a (sema1a*, Haugen *et al.*, unpublished) genes in embryos. We achieve 70% knockdown on average and have obtained up to 90% knockdown of embryonic genes. Knockdown of *Ae. aegypti fra* at these levels has allowed us to phenocopy the *Drosophila fra* mutant phenotype (Clemons *et al.*, in preparation). Our studies suggest that this methodology can be used broadly for targeted disruption of embryonic gene function.

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APPENDIX

RECIPES

MICROINJECTION BUFFER (10x)

Reagent	Quantity (for 500 ml)	Final concentration
KCl	1.86 g	50 mM
NaH_2PO4 (anhydrous)	0.06 g	1 mM

Bring volume to 500 ml with sterile RNAse-free dH_20. Adjust the pH to 7.2 and store at room temperature.

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